Mechanical stimulation activates Gαq signaling pathways and 5-hydroxytryptamine release from human carcinoid BON cells

Minsoo Kim,1 Najma H. Javed,2 Jun-Ge Yu,3 Fievos Christofi,3 and Helen J. Cooke4

1Department of Pharmacology, The Ohio State University, College of Medicine, Columbus, Ohio, USA
2Department of Physiology and Health Science, Ball State University, Muncie, Indiana, USA
3Department of Anesthesiology, and
4Department of Neuroscience, The Ohio State University, College of Medicine, Columbus, Ohio, USA

Address correspondence to: Helen J. Cooke, Department of Neuroscience, 333 West Tenth Avenue, The Ohio State University, Columbus, Ohio 43210, USA.
Phone: (614) 292-5660; Fax: (614) 688-8742; E-mail: cooke.1@osu.edu.

Received for publication February 7, 2001, and accepted in revised form August 21, 2001.

5-Hydroxytryptamine (5-HT) released from enterochromaffin cells activates secretory and peristaltic reflexes necessary for lubrication and propulsion of intestinal luminal contents. The aim of this study was to identify mechanosensitive intracellular signaling pathways that regulate 5-HT release. Human carcinoid BON cells displayed 5-HT immunoreactivity associated with granules dispersed throughout the cells or at the borders. Mechanical stimulation by rotational shaking released 5-HT from BON cells or from guinea pig jejunum during neural blockade with tetrodotoxin. In streptolysin O–permeabilized cells, guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S) and a synthetic peptide derived from the COOH terminus of Gq abolished mechanically evoked 5-HT release, while the NH2-terminal peptide did not. An antisense phosphorothioated oligonucleotide targeted to a unique sequence of Gq abolished mechanically evoked 5-HT release and reduced Gq protein levels without affecting the expression of Gqα11. Depletion and chelation of extracellular calcium did not alter mechanically evoked 5-HT release, whereas depletion of intracellular calcium stores by thapsigargin and chelation of intracellular calcium by 1,2-bis (o-Aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) reduced 5-HT release. Mechanically evoked 5-HT release was inhibited by somatostatin-14 in a concentration-dependent manner. The results suggest that mechanical stimulation of enterochromaffin-derived BON cells directly or indirectly stimulates a G protein–coupled receptor that activates Gq, mobilizes intracellular calcium, and causes 5-HT release.


Introduction

Enterochromaffin cells that store 5-hydroxytryptamine (5-HT) have been linked to several bowel disorders including malignant carcinoid, dumping, and irritable bowel syndromes (IBSs) (1–3). Carcinoid syndrome represents a spectrum of heterogeneous symptoms from vague abdominal complaints to flushing, bronchoconstriction, and diarrhea that result from release of bioactive mediators from carcinoid tumors (4). Although the incidence of carcinoid tumors is relatively low (1.5 per 100,000 population), they comprise about 25% of all small intestinal tumors (4). 5-HT, the major secretory product of carcinoid tumors, is one of the putative mediators of carcinoid diarrhea, which is often relieved by 5-HT receptor antagonists or treatment with somatostatin derivatives (5, 6). The disadvantages to the latter treatment are the broad spectrum of actions and failure of some patients to respond to somatostatin. BON cells are a human carcinoid cell line derived from a metastasis of a pancreatic carcinoid tumor of enterochromaffin cell origin (7, 8). BON cells have characteristics of foregut and midgut tumors and contain a variety of mediators, including neurotensin and pancreastatin in addition to 5-HT (8, 9). Understanding the mechanisms triggering release of 5-HT from BON cells may provide new insights into the biology of carcinoid tumors and into the development of therapeutic interventions for tumors not responding to conventional therapies.

Alteration in 5-HT release is not confined to neoplastic enterochromaffin cells. In a subset of IBS patients, an increased number of nontransformed enterochromaffin cells was associated with an increased sensitivity to transmucosal pressure (1, 9). Selective 5-HT3 receptor antagonists and 5-HT4 agonists have proven to be beneficial in the treatment of diarrhea- and constipation-predominant symptoms, respectively (10–12). Identifying the signaling mechanisms that activate enterochromaffin cells is necessary in understanding the symptoms of several disease states of the gut, as well as its normal physiology. Therefore, the BON cell line provides a unique model to study the mechanosensitive signaling pathways regulating 5-HT release in cells with similarities to nontransformed, intestinal enterochromaffin cells.
Since processes of enteric neurons do not penetrate into the lumen, 5-HT from enterochromaffin cells plays a critical role in activating intrinsic primary afferents that are in reflex pathways regulating secretion and motility (13–18). Although mechanical stimuli are important modulators of cellular function, little is known about mechanisms of mechanotranusduction in enterochromaffin cells. In other cell systems, mechanical stimulation such as pressure, strain, or shear force is associated with activation of quanine nucleotide-binding proteins (G proteins) and an increase in intracellular calcium concentration (19–23).

G proteins are heterotrimeric consisting of α, β, and γ subunits. They are classified by the amino acid sequences of the α subunit into subfamilies (Gαs, Gαi, Gq, and Gα12/13) (24). Upon activation, guanosine S′-diphosphate–bound (GDP-bound) α subunit releases GDP and binds to GTP, causing dissociation of the heterotrimer into the active α subunit and the βγ dimer. The Gq family consists of Gqα, G11α, G14α, G15α, and G16α. They are all regulators of the phospholipase C-β (PLCβ), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes Ca2+ from intracellular stores whereas DAG activates protein kinase C (PKC) (24). Activation of several G protein–coupled receptors, such as muscarinic receptors (M1, M3), adrenoreceptors (β), and purinoceptors (A2), is positively or negatively associated with 5-HT release from enterochromaffin cells (ref. 25 and unpublished data).

The overall aim of this study was to identify mechanosensitive intracellular signaling pathways that regulate 5-HT release. To this end, it was necessary to determine if mechanical stimulation could release 5-HT from the human carcinoid cell line, BON, which has characteristics of enterochromaffin cells and to identify whether G proteins were involved. The results showed that mechanical stimulation activates the Gq subunit, mobilizes intracellular Ca2+, and releases 5-HT.

**Methods**

**Cell culture.** BON cells were kindly provided by C.M. Townsend, Jr. (University of Texas, Galveston, Texas, USA). BON is a noncloned cell line that is a mixed population of large round cells and small cells with dendritic-like extensions. BON cells were subcloned by serial dilution, and clone 7 was chosen on the basis of having the highest content of 5-HT released by the calcium ionophore, A23187 (10 µM). Monolayers of BON cells were maintained in DMEM: nutrient mixture F-12 (DMEM/F12) (1:1) media, supplemented with 10%FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C (8). Cells were trypsinized and seeded at a density of 105 or 106 cells per well in 12-well culture plates (Corning-Costar Corp., Corning, New York, USA). Cells were grown for 48 hours to achieve confluence before experiments, with the exception of the antisense experiments in which cells were grown for 6 days. Passage numbers were 10–30.

**Materials.** Concentrations of drugs were chosen based on published values and from preliminary experiments. Alproclate, pargyline, and thapsigargin were purchased from RBI (Natick, Massachusetts, USA). Tetrodotoxin was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). BAPTA-AM [1,2-bis (o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl) ester] was purchased from Calbiochem-Novabiochem Corp. (San Diego, California, USA). Somatostatin-14 (lot number ZO146) was obtained from Bachem California (Torrance, California, USA). Phosphorothioated oligonucleotides were synthesized and purified by high-performance liquid chromatography by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). Synthetic COOH-terminal peptide (amino acid residues 340 to 359) and NH2-terminal peptide (amino acid residues 13 to 29) of Gqα were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA).

**Antiserum.** The primary Ab was a monoclonal mouse anti–5-HT Ab, clone number M0758, used at a dilution of 1:25 (DAKO Corp., Carpernteria, California, USA). The secondary Ab was a horse anti-rabbit IgG conjugated to FITC (Vector Laboratories, Burlingame, California, USA) used at a dilution of 1:40. The specificity of the 5-HT Ab was determined by preadsorbing with 0.5 mM 5-HT for 1 hour at 37°C. The primary rabbit polyclonal Gq Ab (E-17; Santa Cruz Biotechnology) was raised against a peptide epitope within the amino terminal domain of Gqα of mouse origin. Rabbit polyclonal Gα11 Ab (D-17; Santa Cruz Biotechnology) was raised against a peptide mapped to the amino terminal domain of Gα11 of mouse origin. Both Ab’s are specific and do not cross-react with each other or to other Gα subunit proteins. The primary Ab’s were used in a dilution of 1:2,000. The secondary Ab was a peroxidase-conjugated anti-rabbit IgG used at 1:3,000. Immunopositive bands were visualized with the enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

**Immunofluorescent detection of 5-HT.** Bon cells (105–106) were plated on number 0 coverslips and maintained in DMEM/F12 at 37°C in a 5% CO2 incubator for 3 days. After fixation for 1 hour at 4°C in modified Zamboni’s fixative, the cells were washed three times at 10-minute intervals with PBS. The cells were incubated in normal horse serum (3%) in 0.5% Triton-X/PBS for 30 minutes at room temperature before an overnight incubation with the 5-HT Ab. The secondary Ab was conjugated to FITC at 4°C. The cells were washed with PBS three times at 10-minute intervals and incubated for 2 hours at room temperature with the secondary Ab, a horse anti-mouse IgG. A third and final PBS wash was performed. The specificity of the 5-HT Ab was determined by preadsorbing with 0.5 mM 5-HT for 1 hour at 37°C. Cells were visualized by laser-confocal imaging using the Zeiss LSM 410 microscope (Carl Zeiss Inc., Thornwood, New York, USA). The cells were excited with an
Ar-Kr laser at 488 nm. Emissions were captured by a photomultiplier tube through a LP 590 filter and saved on an IBM computer as Tagged Image File Format (TIFF) images. The thickness of each optical slice was 0.5–1.5 µm. Images represent averages of four scans.

**Electron microscopy.** BON cells were fixed onto 35-mm dishes (Corning-Costar Corp.) using 1.6% glutaraldehyde, 0.4 mM CaCl₂, and 0.05 M sucrose in 0.1 M PBS, pH 7.4, for 30 minutes at 4°C. The cells were rinsed in 0.1 M PBS, pH 7.4, containing 0.1 M sucrose, post-fixed in 1% osmium tetroxide, and dehydrated through an ethanol series (50%, 70%, 80%, 95%, 100%). Cells were rinsed in hydroxypropylmethacrylate for 30 minutes followed by rinsing in three changes of Polybed 812 resin overnight at 60°C. Approximately 800-nm sections were cut using a Reichert Ultracut E Ultramicrotome, placed on copper grids, and stained with 2% uranyl acetate and Reynold’s lead citrate. Specimens were observed in a Philips CM 12 transmission electron microscope at 60 kV.

**Mechanical stimulation.** The medium was removed before washing the cells with Earle’s balanced buffer solution (EBBS) (pH 7.3) containing 0.1% BSA. After preincubation for 30 minutes at 37°C, the medium was removed and replaced with 0.5 ml EBBS that contained 10⁻⁵ M of alaproclate (5-HT uptake inhibitor) and pargyline (monooamine oxidase inhibitor). The cells were exposed to static conditions or to a mechanical stimulation over the trypsin-like and cysteine protease inhibitor and trypsin inhibitors, respectively, and in addition the trypsin-like and cysteine protease inhibitor 1 µM leupeptin. The resuspended pellets were incubated for 30 minutes on ice after sonication and insoluble material was removed by centrifugation (10,000 g for 3 minutes at 4°C). Protein concentration of each well was determined (Bio-Rad, Hercules, California, USA) according to the manufacturer’s instructions. The sensitivity of the assay was confirmed by the manufacturer. About 0.2 × 10⁴ to 2 × 10⁴ cells/well were sufficient for most experimental setups.

**5-HT enzyme immunoassay.** 5-HT release was measured by enzyme immunoassay using an ELISA kit (Coulter Corp., Miami, Florida, USA) according to the manufacturer’s instructions. The absorbance was measured at 405 nm and 5-HT concentration was determined from a standard curve. The sensitivity of the assay is approximately 0.5 nM (1.8 pg/well). With a sample range between 4.5 and 5.5 nM (n = 30), the interassay standard deviation and coefficient of variation were 0.4 nM and 8.9%, respectively.

**Immunoblotting.** The frozen cells were thawed and gently scraped from culture plates with a cell scraper. After centrifugation (3,000 g for 5 minutes at 4°C), pellets were resuspended in lysis buffer. It contained 10 mM Tris-HCl, pH 8.0, the detergents 1% Triton X-100 and 60 mM octyl glucose, 150 mM NaCl, 5 mM EDTA, 500 µM 4-(2-Aminoethyl) benzzenesulfonylfluoride-HCl, 1 µM E-64, 150 mM apronin, which are serine, cysteine, and trypsin inhibitors, respectively, and in addition the trypsin-like and cysteine protease inhibitor 1 µM leupeptin. The resuspended pellets were incubated for 30 minutes on ice after sonication and insoluble material was removed by centrifugation (10,000 g for 3 minutes at 4°C). Protein concentration of each well was determined (Bio-Rad, Hercules, California, USA). Solubilized proteins (29 µg) were separated by 10% denaturing SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The α subunits of G proteins were detected by immunostaining with rabbit
polyclonal Ab’s against Gαq or Gα11. Nitrocellulose membranes were blocked with 5% nonfat milk for 2 hours at 37°C and probed with primary Ab’s (1:2,000) for 1 hour at 37°C. Membranes were rinsed with PBS/Tween-20 (80 mM Na2HPO4 • 7H2O, 20 mM KH2PO4, 100 mM NaCl, 1% [vol/vol] Tween 20, pH 7.5) and incubated with peroxidase-conjugated anti-rabbit IgG at 1:3,000 dilution for 30 minutes at room temperature. Immunopositive bands were visualized with the ECL kit (Amersham Pharmacia Biotech). The protein bands visualized on Hyperfilm ECL (Amersham Pharmacia Biotech) were scanned, and the intensity of each band was measured by ScanWizard 5 software (Microtek Lab Inc., Redondo Beach, California, USA).

Statistics. All data are expressed as means plus or minus standard errors of the means (SEM). Statistical significance was evaluated by paired or unpaired Student’s t test or ANOVA with Bonferroni’s multiple comparison post hoc test, depending on experimental design.

Results

Electron microscopy. BON cells were subcloned by serial dilution and clone 7 was chosen on the basis of having the highest releasable content of 5-HT. BON cells were characterized by membrane protrusions reminiscent of microvilli in enterochromaffin cells (27). (Figure 1, a and b). On the side attached to the culture plate, membrane protrusions were very rarely seen (Figure 1, b and c). BON cells contained a large number of dense-core secretory granules distributed throughout the cell or localized at one pole of the cell often associated with microvilli (Figure 1, b and d).

5-HT immunoreactivity in BON cells. 5-HT immunoreactivity indicated by the white color was evident in nearly all BON cells (>98%) (Figure 2, a, b, d, and e). 5-HT immunoreactivity was present in granules (Figure 2, d and e). Preadsorption of the 5-HT Ab with 5-HT abolished any positive reaction, indicating that the Ab is specific for 5-HT (Figure 2, c and f).

5-HT release from BON cells in response to secretagogues. Ca2+ ionophore A23187, which allows entry of Ca2+ into cells from extracellular sources, and forskolin, which stimulates adenylyl cyclase to increase cAMP production, caused a very large increase in 5-HT release compared with basal 5-HT release (control: 0.9 ± 0.1 pmol/well/20 min) (Table 1). In contrast, receptor-mediated 5-HT release was less, as illustrated by isoproterenol, which activates β-adrenergic receptors, Gαs, adenylyl cyclase, and cAMP, and bethanechol, which activates muscarinic receptors, Gαq, IP3, and calcium or a Gαi pathway (Table 1).

Effect of mechanical stimulation on 5-HT release. To determine whether mechanical stimulation by rotational shaking would enhance 5-HT release from enterochromaffin cells in vitro, whole-thickness preparations of the guinea pig small intestine (jejunum) were exposed to increasing speeds of shaking. The assay buffer contained 500 nM of tetrodotoxin to prevent neuronally evoked 5-HT release. Increasing the shaking speed from 150 to 250 rpm caused a graded increase in 5-HT release (Figure 3a).

BON cells exposed to mechanical stimulation by shaking the culture plates on an orbital shaker at...
increasing rpm from 50 to 100 for 20 minutes showed an rpm-dependent increase in 5-HT release (Figure 3b). Significant increases in 5-HT release above controls (0 rpm) occurred at 60 rpm (157% ± 24%) with a maximum at 80 rpm (211% ± 29%). Cell viability after shaking, assessed by trypan blue exclusion, total protein assay, cell numbers, and lactate dehydrogenase (LDH) release, were not significantly different from the control group (Table 1).

**Effects of streptolysin O on LDH activity and 5-HT release.**

There was a sharp increase in LDH activity, a marker of plasma membrane integrity, within the first 5 minutes after exposure to streptolysin O (SLO), followed by a more gradual rise with time after SLO wash out (Figure 4a). 5-HT release in static controls and in mechanically stimulated cells gradually declined in parallel as a function of time after SLO treatment (Figure 4b). Despite the high activity of LDH 115 minutes after permeabilization with SLO and the gradual decrease in basal and mechanically stimulated 5-HT release, the cells were still able to respond to mechanical stimulation by an increase in 5-HT release similar to nonpermeabilized cells. The decrease in 5-HT release in the basal state paralleled that in the stimulated states (Figure 4b). Linear regression analysis showed that the slopes are not significantly different (–0.018 ± 0.0050, 0 rpm, and –0.032 ± 0.0098, 80 rpm; P = 0.21) under static or stimulated conditions.

**Role for G proteins in mechanically evoked 5-HT release.**

There was no detectable change in basal 5-HT release from permeabilized cells compared with nonpermeabilized cells in the static or stimulated condition. Pretreatment of permeabilized BON cells with GDP-β-S (200 µM) abolished mechanically evoked 5-HT release without affecting basal release (Figure 5a). To investigate the role of Gqα in mechanically evoked release of 5-HT, BON cells were treated with a phosphorothioate-antisense oligonucleotide derived from a unique sequence of Gqα. Controls consisted of untreated cells or cells exposed to an oligonucleotide with a scrambled sequence. The latter had the same guanine/cytosine content as the antisense oligonucleotide and the nucleotides were arranged in random order. Treatment of the cells with 1 µM antisense oligonucleotide for 6 days did not alter basal 5-HT release. Mechanical stimulation increased 5-HT release in both control groups (Figure 6a). However, in antisense oligonucleotide-treated cells, mechanical stimulation did not increase 5-HT release (Figure 6a). Inhibition of mechanically evoked 5-HT release occurred without compromising functional integrity of the 5-HT exocytosis/release mechanisms. Ability to release 5-HT was assessed by treatment of the cells with a calcium ionophore (10 µM A23187). A23187 evoked a similar release of 5-HT in pmol/µg protein/well/20 min for scrambled oligonucleotide-treated cells (0 M, 0.3 ± 0.1 [n = 5]; 10 µM, 9.8 ± 0.2 [n = 5]) and antisense oligonucleotide-treated cells (0 M, 0.5 ± 0.1; 10 µM, 8.3 ± 0.5).

The results of Western blot analysis indicated that the antisense oligonucleotide targeting Gqα reduced expression of Gqα protein compared with control and scrambled oligonucleotide-treated cells (Figure 6, b and

---

**Table 1**

Secretagogue-evoked 5-HT release and cell viability after shaking

<table>
<thead>
<tr>
<th>Drug</th>
<th>Signaling path</th>
<th>10 µM secretagoues/5-HT release</th>
<th>S-HT release</th>
<th>Assay</th>
<th>Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionophore (%)</td>
<td>Ca² influx</td>
<td>1,910 ± 645 (3)</td>
<td>0.9 ± 0.1/0.7 ± 0.1 (4)</td>
<td>LDH (%)</td>
<td>80 rpm/0 rpm</td>
</tr>
<tr>
<td>Forskolin (%)</td>
<td>AC/cAMP</td>
<td>2,104 ± 453 (3)</td>
<td>8.7 ± 1/8.4 ± 1 (7–8)</td>
<td>Trypan blue (%)</td>
<td></td>
</tr>
<tr>
<td>Isoproterenol β-R (%)</td>
<td>Geys, AC/cAMP</td>
<td>357 ± 99 (3)</td>
<td>3.1 ± 0.1/3.5 ± 0.1 (4-10)</td>
<td>Protein (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>Bethanechol M1/ M1-R (%)</td>
<td>Gqα, IP3, Ca²</td>
<td>179 ± 21 (3)</td>
<td>1.1 ± 0.03/1.0 ± 0.04 (7–8)</td>
<td>Cell no. x 10⁶</td>
<td></td>
</tr>
</tbody>
</table>

Means ± SEM are shown. 5-HT release is percent control value, 0.9 ± 0.1 pmol/well/20 min. Numbers in parentheses are n values. AC, adenyl cyclase.

---

*Figure 3*

Effect of mechanical stimulation on 5-HT release from guinea pig small intestine (a) and human BON cells without (b) and with somatostatin-14 (c). Tissues and cells were maintained in a static condition (0 rpm; control) or exposed to mechanical stimulation ranging from 50 to 100 or 150 to 250 rpm for 20 minutes at 37°C. Values represent 5-HT release relative to controls in picomoles per well per 20 minutes. (b) Cells 0.8 ± 0.08. (c) Cells 1.3 ± 0.1. (a) Tissue 9.8 ± 4. Increasing rpm resulted in rpm-dependent increase in 5-HT release (n = 32, 0 rpm; n = 4, 50 rpm; n = 10, 60 rpm; n = 6, 80 rpm; n = 16, 100 rpm). *P < 0.05 versus 0 rpm.
Effects of SLO on LDH activity and 5-HT release. BON cells were permeabilized by 20 U/ml SLO for 5 minutes at 37°C in permeabilizing buffer. After permeabilization, SLO was washed out, and BON cells were incubated at 37°C for different time periods from 5 minutes to 115 minutes. (a) Buffers were collected after each time period and the amount of LDH activity was measured. Values represent percentage of total LDH activity (n = 3). (b) After incubations, BON cells were stimulated with/without rotational shaking (80 rpm) and 5-HT release was measured after the stimulation (20 minutes). Values represent concentrations of 5-HT in wells. Linear regression analysis of 5-HT release showed that the slopes were not significantly different (-0.018 ± 0.0050 and -0.032 ± 0.0098; P = 0.21) under static or stimulated conditions (80 rpm; n = 3).

c. Studies were done to ensure that the amount of protein loaded in each lane was the same and the antisense treatment affected only Gqα rather than Gqβ11, which has high amino acid sequence homology (88%) with Gqα. The immunoblot was stripped and reprobed with polyclonal Ab's specific for Gqβ11. The results indicate that Gqβ11 is still expressed at the same levels in control and after Gqα antisense oligonucleotide treatment (Figure 6, a and c).

Studies were done to investigate whether the synthetic COOH-terminal peptide (residues 340–359, VFAAVKDTILQLNLKEYNL) of Gqα would affect shaking-evoked release of 5-HT. In SLO-permeabilized BON cells, pretreatment of 25 µg/ml of COOH-terminal peptide for 5 minutes abolished shaking-evoked 5-HT release (Figure 5b). On the other hand, the NH2-terminal peptide (residues 13–29, EEKAERINDEIERQL) of Gqα had no significant effect on 5-HT release (Figure 5b).

Effect of Ca2+-free buffer, Ca2+ chelators, or depletion of intracellular Ca2+ stores on basal and mechanically evoked 5-HT release. The next series of studies were designed to assess the involvement of Ca2+ in mediating mechanically evoked release of 5-HT. Ca2+ ionophore A23187 (Table 1) and thapsigargin, which releases intracellular Ca2+, significantly increased 5-HT release compared with controls (control: 0.9 ± 0.1 pmol/well/20 min; 1 µM thapsigargin, 2.1 ± 0.6 pmol/well/20 min; P < 0.05, n = 3-6).

Since these results suggested that 5-HT release by BON cells is Ca2+-dependent, studies were done to determine the role of extracellular and intracellular Ca2+ pools in mechanically evoked 5-HT release. In Ca2+-free EBBS containing the extracellular chelator 1 mM EGTA, basal 5-HT release was not different from control (calcium-containing buffer: 0.8 ± 0.08 pmol/well/20 min; Ca2+-free buffer: 0.9 ± 0.1 pmol/well/20 min) (Figure 7a). At this concentration of EGTA there was no apparent cell detachment. In the absence of extracellular Ca2+, mechanical stimulation (80 rpm) significantly increased 5-HT release. The relative enhanced rate, 176% ± 10% of static control, was not significantly different from the rate in Ca2+-containing buffer (211% ± 29%) (Figure 7a).

Because mechanical stimulation can induce 5-HT release in the absence of extracellular Ca2+, additional experiments were performed to investigate the possibility that mechanical stimulation mobilizes Ca2+ from internal Ca2+ stores. To investigate this possibility, the cells were pretreated with high concentrations of thapsigargin (10 µM), an inhibitor of the endoplasmic reticular Ca2+-ATPase, to deplete intracellular Ca2+ stores. Thapsigargin pretreatment inhibited shaking-evoked 5-HT release by 75% (Figure 7b). Second, BON cells were pretreated with cell-permeable Ca2+ chelator, BAPTA-AM (50 µM), for 1 hour to buffer internal Ca2+ levels before being tested in Ca2+-free EBBS. Mechanically evoked (80 rpm) 5-HT release was completely abolished by BAPTA-AM pretreatment (111% ± 5% of the static value) (Figure 7c). Taken together, these data strongly indicate that shaking mobilizes Ca2+ from intracellular stores.

Effect of somatostatin on 5-HT release. Because somatostatin derivatives are often used therapeutically in patients with carcinoid syndrome, we examined the effect of somatostatin-14 on 5-HT release. Somatostatin-14 had no effect on basal release (0 rpm: 1.3 ± 0.1 pmol/well/20 min; 0 rpm + 10 µM somatostatin-14: 1.0 ± 0.04 pmol/well/20 min). However, this peptide significantly inhibited mechanically stimulated 5-HT release in a concentration-dependent manner (Figure 5a).

SLO-permeabilized BON cells. Cells were permeabilized by 20 U/ml SLO for 5 minutes in permeabilizing buffer and preincubated with 200 µM of GDP-β-S for 5 minutes before stimulation (shaking at 80 rpm): 0 rpm, 2.9 ± 0.4 pmol/well/20 min; 0 rpm + GDP-β-S, 3.1 ± 0.3 pmol/well/20 min. *P < 0.05 versus permeabilized static control, †P < 0.05 versus shaking at 80 rpm of permeabilized cells (n = 9). (b) BON cells were permeabilized by 20 U/ml SLO for 5 minutes in permeabilizing buffer and preincubated with 25 µg/ml of NH2-terminal peptide (GqN17) or COOH-terminal peptide (GqC20) for 5 minutes before mechanical stimulation at 80 rpm. Experiments were performed in the presence of 1× protease inhibitor cocktail. 5-HT release in pmol/well/20 min was 0 rpm, control: 2.2 ± 0.02; GqN17: 1.8 ± 0.4; GqC20: 2.1 ± 0.07. *P < 0.05 versus permeabilized static control, †P < 0.05 versus shaking at 80 rpm of permeabilized cells (n = 3).
At a concentration of 10 µM, somatostatin-14 abolished mechanically evoked 5-HT (Figure 3c).

**Discussion**

Our results indicate that mechanical stimulation by rotational shaking causes 5-HT release from the small intestine of the guinea pig even when neural conduction was blocked. While mechanical stimulation releases 5-HT independent of any neural input, it is unclear if this is a direct effect on enterochromaffin cells or whether it is due to release of other mediators from a variety of cell types found in tissue preparations. This possibility could explain why 5-HT release was considerably greater in tissues compared with BON cells. The number of cell types found in tissues was minimized by using BON cells that displayed similarities to non-transformed enterochromaffin cells. Clone BON7 was relatively homogenous in that nearly all the cells (> 98%) contained 5-HT immunoreactivity localized to granules (Figure 2). Granules were often associated with one pole of the cell, as has been reported for enterochromaffin cells in vivo (27).

The factor that transduces a mechanical force into a biological response in BON cells is unknown. However, the protrusion of microvilli into the buffer where they can “taste” the contents suggests that they may contain mechanotransducing elements analogous to hair cells (28). BON cells also have other sensory modalities by virtue of their chemosensitivity to hexoses (29).

Rotational movement of assay buffer in culture plates generates several mechanical forces, including shear stress or continuously changing hydrostatic pressure gradients, or both (30). In vivo, shear stress is likely to occur in the small intestine and proximal colon where the luminal contents are fluid, or in the distal colon when they become fluid such as in diarrheal states. Mechanical stimulation generated by direct contact of the enterochromaffin cells with the luminal contents occurs frequently throughout the intestinal tract. Others have reported that balloon distention, puffs of nitrogen gas ejected from a pipette, and stroking with a brush evoke 5-HT release (31, 32). Many of these stimuli are composed of several different forces acting simultaneously. Thus in the continuously changing environment...
environment of the bowel, composite stimuli may more readily reflect the in vivo condition.

Our results suggest that rotational shaking of cultured BON cells activates heterotrimeric G proteins causing 5-HT release. Based on published reports, it seemed likely that the Gq family, which consists of Gq11, Gq14, Gq15, and Gq16, might be involved. Gq14, Gq15, and Gq16 are unlikely candidates because they are found in epithelial, stromal, or hematopoietic cells. Evidence for Gq in BON cells comes from several different experimental regimens in which stimulated 5-HT release was reduced or abolished: (a) GDP-β-S, which prevents activation of heterotrimeric G proteins inhibited 5-HT release; (b) an antisense oligonucleotide directed against a unique sequence of human Gq cDNA reduced expression of Gq protein and abolished mechanically evoked 5-HT release without decreasing cell density, total cell protein, cellular responsiveness to a calcium ionophore; and (c) the synthetic COOH-terminal peptide of Gq abolished 5-HT release.

One puzzle is the observation that the antisense treatment reduced Gq protein levels by 50%, whereas it abolished mechanically evoked 5-HT. Our results are compatible with the possibility that there is more than one pool of Gq in BON cells. In endothelial cells, most of the Gq associates with caveolin in membrane invaginations called caveolae (33).

The effect of the antisense oligonucleotides in abolishing 5-HT release was unlikely to be due to impairment of the vesicular monoamine transporters, VMAT1 and VMAT2, and a reduction of 5-HT content in secretory vesicles. Whereas these transporters in carcinoid tumors are regulated by pertussis toxin-sensitive Go2, mechanically stimulated 5-HT release is mediated by Gq that is resistant to pertussis toxin.

The COOH-terminus of Gq encodes an intracellular domain that interacts with the agonist-occupied, G protein–coupled receptors (34). Because the C termini of Gq and Gq11 have considerable sequence homology, the synthetic COOH-terminal peptide does not discriminate between the two. This is not problematic, because the antisense experiments described above targeted a sequence that was unique to Gq, and therefore they provide strong evidence that the Gq pathway is involved in mechanically evoked 5-HT release. On the other hand, the purpose of the experiments with the synthetic peptide was to test whether it could disrupt the coupling of the G protein with its receptor by competing with the endogenous domain of Gq as described for Gq-coupled α1B adrenergic receptor-mediated signaling (35). The finding that antisense oligonucleotides abolished mechanically evoked 5-HT release coupled with the observation that the COOH-terminal peptide of Gq also abolished 5-HT release suggests that a ligand-occupied receptor is coupled to Gq during mechanotransduction in BON cells. Consistent with the conclusion that mechanical stimulation activates a G protein–coupled receptor is the observation in endothelial cells that fluid flow increased mRNA levels of FEB-1 and edg-1, two G protein–coupled receptor genes (36).

All Gα subunits are modified at or near their NH2-termini by covalent attachment of the fatty acids myristate and/or palmitate that support membrane association and signaling of G proteins (24). Therefore, mechanical stimulation of the phospholipid bilayer could activate G proteins directly without the necessity of an agonist-occupied receptor (23). Indeed, shear stress applied to reconstituted phospholipid liposomes increased GTPase activity of Gq and Gq11 under conditions when neither receptor proteins nor cytoskeletal proteins were present. While our results cannot completely rule out the possibility that the mechanical stimulus activates the phospholipid bilayer/G protein complex directly, studies with the COOH-terminal peptide suggest that this is unlikely. Since the synthetic peptide that interferes with the coupling of the G protein with the receptor completely abolished 5-HT release in BON cells, there was no residual 5-HT release to attribute to another mechanism.

Although our results rule out the possibility that paracrine substances from other cell types mediate mechanically evoked 5-HT release; they do not eliminate autocrine mediators. Indeed, mechanical stimuli such as shear, tensile, and osmotic stress were shown to increase ATP release, which then can activate P2 receptors (37–39). It is unknown whether P2 receptors are present on BON cells, although they have been reported to inhibit 5-HT release from enterochromaffin cells (25).

Regardless of the mechanism, activation of Gq leads to elevation of intracellular Ca2+, which triggers 5-HT release (25). An increase in intracellular Ca2+ due to influx of extracellular Ca2+ through voltage-gated Ca2+ channels on the plasma membrane is not essential for mechanically evoked 5-HT release in BON cells, whereas Ca2+ mobilization from intracellular stores is a necessity. This conclusion is supported by the finding that 5-HT is still released under Ca2+-free conditions. Furthermore, this conclusion is reinforced by the findings that 5-HT release was inhibited by depletion of intracellular Ca2+ stores and buffering free Ca2+ in the cytosol. Elevation of intracellular Ca2+ concentration has been shown to be due to release from Ca2+ stores by an activation of IP3 receptors (40). In endothelial cells, shear stress generated by fluid flow activated PLCβ, which hydrolyses PIP2 into IP3 (41), Phosphoinositide hydrolysis is considered to be mediated by either protein tyrosine phosphorylation of PLCγ or G protein activation of PLCβ (42).

In summary, we have devised a noncytotoxic, mechanical stimulus for releasing 5-HT from BON cells. The results indicate that detection of mechanical stimuli by BON cells involves receptor-Gq coupling and mobilization of intracellular Ca2+. These novel findings in BON cells as a model for studying mechanotransduction and 5-HT release begin to uncover the cellular and molecular events that transform mech-
physical forces into biological responses. Drugs targeting the GqG-coupled signaling pathway to facilitate or inhibit 5-HT release would be important therapeutic interventions in disease states where improper sensation and function of enterochromaffin cells cause symptoms of diarrhea or constipation.

Acknowledgments

We would like to thank Helen Raybould for review of the manuscript and helpful comments and Courtney M. Townsend, Jr. for providing human carcinoid BON cells. This work was supported by the NIH grants DK-37240 and DK-57016 (H.J. Cooke) and DK-44179 (F. Christofi).